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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Prostate cancer (PCa) is most commonly diagnosed and second leading cause of death in the US men, representing 8% of all male cancer deaths. Current gold standard for clinical detection of PCa consist of serum PSA test together with digital rectal examination (DRE). However, PSA screening predicted lower sensitivity (~21%) for PCa, while DRE is mostly linked with the patient discomfort due to painful procedure with a risk of slight bleeding and more importantly, poor sensitivity and specificity. Considering such limitations, there is an urgent need for a novel, non-invasive serum-based biomarker in conjunction with high-sensitive technique to differentiate indolent vs. aggressive prostate cancer (PCa). Emerging evidence suggests that several micro RNAs (miRs) are differentially expressed in tissues and sera of PCa patients and can potentially serve as biomarkers for disease progression and aggressiveness. However, quantification of ultra-low levels of serum miRNAs remains difficult with conventional quantification methods. Hence, present study was aimed at developing, optimizing and testing a novel DNA-gold nanoprobe (DNA-AuNPr)-based fluorescence assay for highly sensitive and specific quantification of miRNAs in serum samples. Herein, we successfully synthesized and characterized fluorescently-labeled DNA-gold nanoparticle probes (Nanoprobe) via bioconjugation approach. In parallel, metaanalysis and qPCR analysis were performed on prostate tissues excised from PTEN PCa mouse model and on PCa patient serum to identified differential expression level of miRNAs. We also analyzed expression patterns of various miRNAs (identified through meta-analysis of previously published reports) in mouse PCa tissues and in PCa patient serum compare to their respective controls. We observed a significant overexpression of miR-21 and miR-375 in both case. Further, using our nanoprobe-based fluorescent assay, we were successfully able to quantify an aberrantly expressed miRNAs level in human serum samples. We expect that the nanoprobe-based miRNA detection platform will form the foundation for early detection of miRNA biomarker and differentiation between androgen dependent and independent PCa.

15. SUBJECT TERMS
DNA-Gold nanoprobe, microRNA quantification, Prostate cancer diagnosis, fluorescence assay

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1) Introduction

Developing minimally invasive blood/serum-based test is extremely appealing early stage screening and detection of cancer comparing with highly invasive methods including rectal examinations, tissue biopsies etc. Among many cancer types, Prostate cancer (PCa) is the second leading cause of cancer-related deaths in the US men. According to year 2017 report, PCa accounts for 161,360 new cases of PCa have been diagnosed and around 26,730 estimated new deaths [1, 2] and this accounts for 8 % of all cancer-related deaths among the US men [1]. Hence, an effort for early detection of PCa should be undertaken to improve the survival rate of patients. Currently, prostate-specific antigen (PSA) screening combined with digital rectal examination (DRE) are the gold standard method for monitored and management of PCa screenings. Serum PSA level more than 2.5-4 ng/mL and abnormalities observed during DRE may indicate the presence of PCa. In contrast, even in non-cancerous prostate disease conditions, (i.e. patients with prostatic or benign prostatic hyperplasia (BPH)) can also have increased serum PSA level, resulting in a high rate of prostate biopsies and overtreatment. Therefore, there is an urgent need to introduce high sensitive detection technology to identify novel, more specific and sensitive, non-invasive PCa biomarkers to increase an accuracy of PCa diagnosis and to differentiate between androgen dependent (AD) to androgen independent (AI) PCa. Several previous literature reports have been shown the importance of circulating miRNAs (Cir-miRNAs)[3] as a promising biomarker because of their altered expression in various cancers including PCa [4, 5]. Similarly, other studies focusing the differential expression patterns of miRNAs have also been reported in AD and AI PCa [6]. Considering all these studies, miRNAs could be an ideal candidate to study PCa pathogenesis, to identify early diagnosis markers and to differentiate between aggressive PCa to non-aggressive disease. However, sensitive detection of miRNAs level in the body fluids (blood, urine etc.) tissue samples is remains difficult process, because of lack of selective and sensitive methods. Moreover, conventional methods (i.e. qRT-PCR, next generation sequencing, Northern blot, etc.) have shown some limitations i.e. weak or moderate sensitivity, less specificity, expensive instrumentation, extreme care to avoid contamination, etc. [7]. Therefore, there is an immediate requirement of novel diagnostic platform to identify novel differentially expressed miRNAs in the body fluids (blood, urine, etc.) for an early detection of PCa. Advances in nanotechnology and availability of multiple types of nanomaterials have led to the development of sensitive assay platforms to the novel miRNA based markers for early diagnosis [8]. In past decades, research was directed to develop several nanoscale probes, alongside the discovery of the wide range of biomarkers to lower the detection limit of biomarkers. Therefore, in current proposal our goal is to establish a sensitive detection platform for detection of novel PCa biomarker (i.e. miRNA) for early diagnosis of PCa, distinguishing indolent vs. aggressive and hormone-dependent vs. independent PCa. Here, we have proposed the development of well-characterized gold nanoparticles (AuNPs)-based miRNA detection probes (i.e. Au-DNA nanoprobes) implemented for direct (without converting into cDNA) and sensitive detection of altered miRNA level during PCa progression, which can enable differentiation between aggressive and indolent cancer. Therefore, three different goals were laid out for the study. 1) Development of sensitive gold nanoprobes for direct and sensitive detection of miRNAs 2) Investigate stage specific

global miRNA expression profiles in the PCa mouse tissue followed by their detection in mouse serum using nanoprobes. 3) To investigate the diagnostic potential of differentially expressed miRNAs identified in mouse model and their relevance in sera of human prostate cancer patients.

2) Keywords

Gold nanoprobe, microRNA quantification, Prostate cancer diagnosis, fluorescence assay.

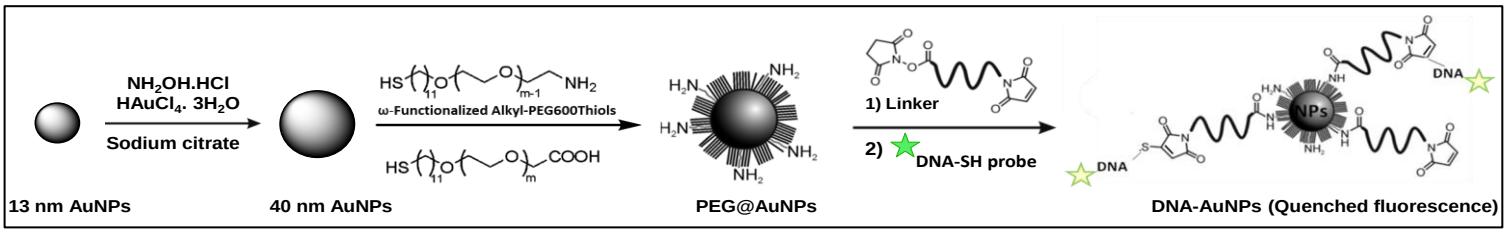
3) Accomplishments

During the previous year, with the funding support from Department of Defense, we have performed the proposed experiments as per the approved statement of work (SOW) and have made following accomplishments.

Major goals 1: Development of fluorescent-tagged-gold nanoprobe (DNA-AuNPs) based sensitive probes and their use for quantification of miRNA (include standardization of assay conditions).

Subtask-1: Synthesis and characterization of miR-21 and miR-375 nanoprobe

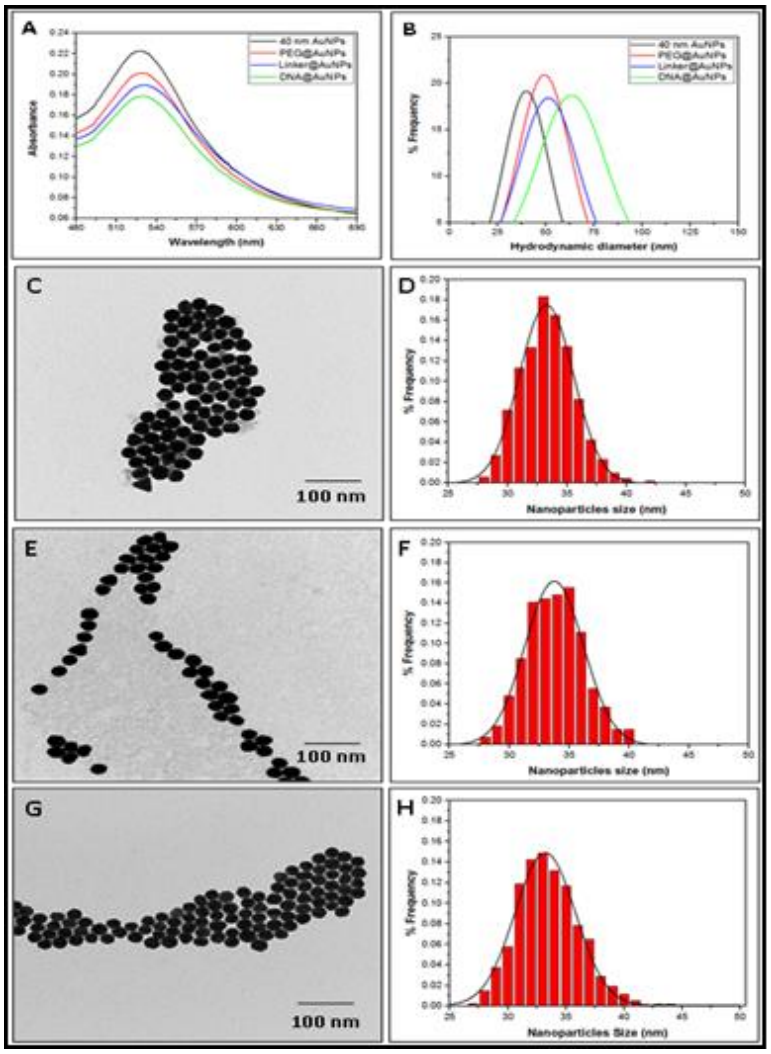
Synthesis, detailed characterization of metal nanoparticles [i.e. Average ~ 40 nm gold nanoparticles (AuNPs)], PEG passivation of AuNPs, Linker reaction and finally making of DNA-AuNPs probe for miR-21 and miR-375.
Making of nanoprobe was initiated with preparation of gold nanoparticle (Size ~40 nm) by growing the 13 nm seed AuNPs, which prepared by well-known Grabar synthesis. We have performed detailed analysis of same particles, their core size and surface charge via DLS, TEM and zeta potential respectively. Further, we followed steps i.e. PEG passivation, Linker binding and DNA-probe conjugation using the established protocols (shown in sceme-1 as below). In details, passivated AuNPs were used to conjugate miR-21 and miR-375 probe DNA via an $SM(EG)_2$ linker and were tested via gel electrophoresis after complete purification. Fluorescence quantitation were used determine the number of probe attached to DTT treated AuNPs.

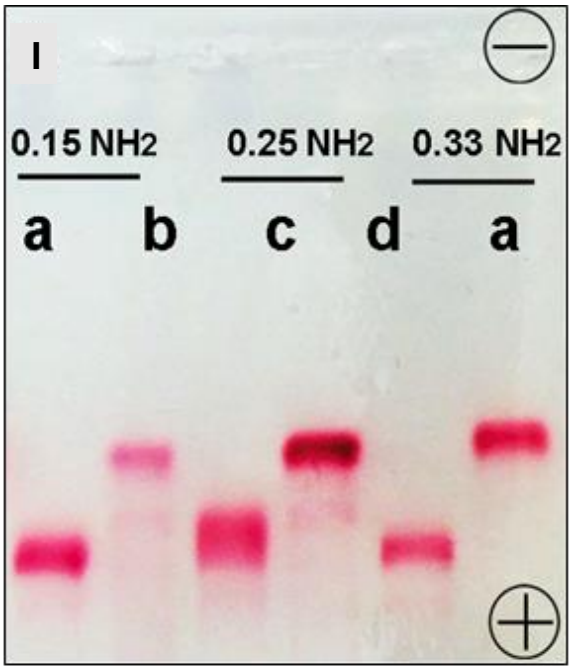


Scheme 1: Synthesis of PEGylated AuNPs functionalized with fluorescently labeled DNA probes (nanoprobe) via Linker approach.

Subtask-2: Characterization of AuNPs and probe

We perform detail analysis of our Au nanoprobe as well as PEG passivated and linker bound AuNPs using standard characterization techniques. Detail interpretation of UV-visible and DLS data indicating red shift in UV spectrum as well as increment in the hydrodynamic diameter of AuNPs. Further, actual size and quality of AuNPs were determined by transmission electron microscopy (TEM) imaging as shown in following figure 1(C, E, G). Later, final bioconjugated product i.e. nanoprobe was checked and verified by gel electrophoresis (Figure 1I). Quantification of number of FAM-labelled probe per single NP was calculated using fluorescence based assay and was calculated through following formula *(N_{Oligo}= [Conc. of FAM] / [Conc. of AuNPs]).* Summary of the calculation given in table 2.





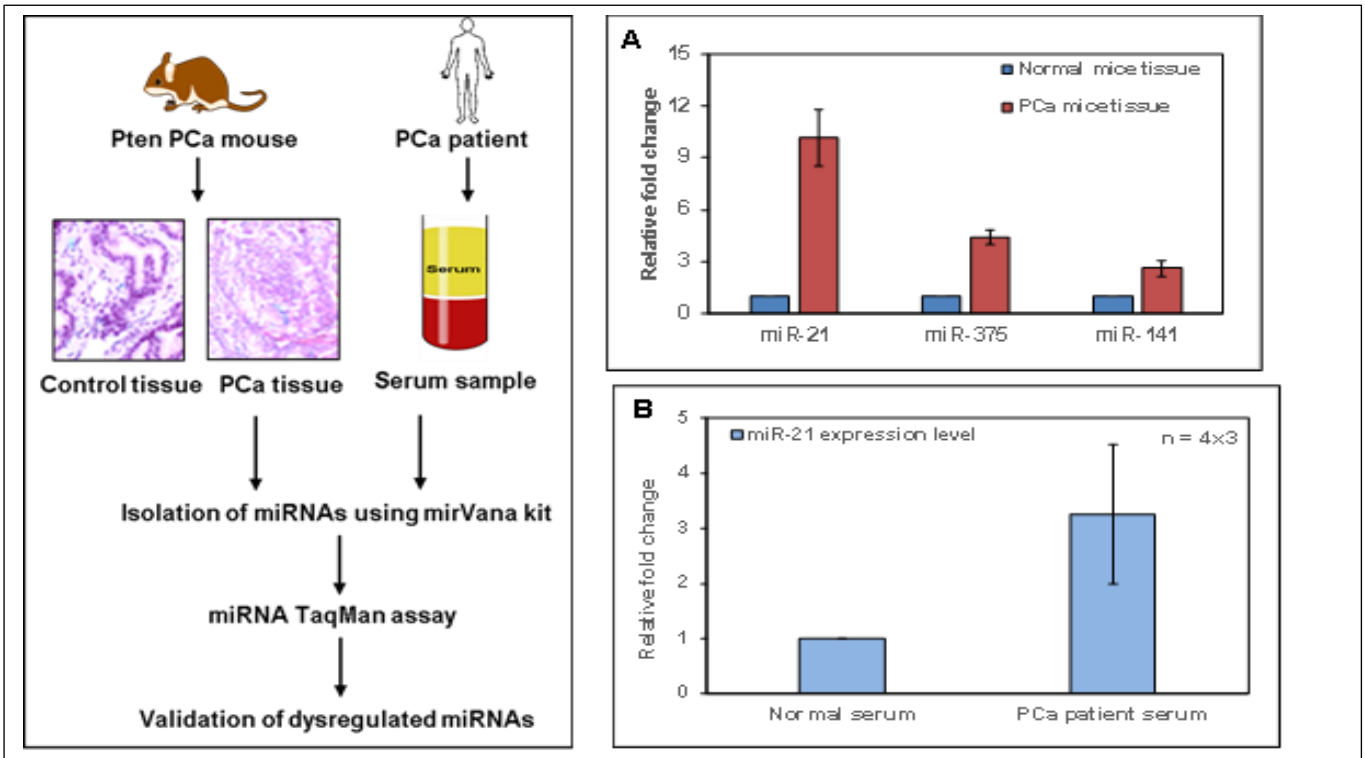
AuNPs type	Mole fraction (χ) of HS-PEG-NH_2	[FAM] nM	[AuNPs] nM	$N_{oligo's}$/NP
DNA@AuNPs	0.15	522.02	2.98	~ 175 ± 15
DNA@AuNPs	0.25	1135.2	7.45	~ 152 ± 21
DNA@AuNPs	0.33	989.92	4.77	~ 207± 19

Figure 1: Images (A & B) represent UV-vis and DLS spectrums of bare, PEGylated, Linker and DNA-conjugated AuNPs respectively. TEM images and SDH of AuNPs i.e. respectively PEG@AuNPs (C & D), Linker@AuNPs (E & F), DNA@AuNPs (G & H). Image (A) Gel electrophoresis proving successful DNA-AuNP conjugation. Reduced electrophoretic mobility of the AuNPs (Lanes b, d, f) after the DNA conjugation compare to the linker@AuNPs (Lanes a, c, e) shown successful immobilization of DNAs on AuNPs surface.

Major task 2: Application of well-developed DNA-NP nanoprobes for examination of the specific global miRNA expression profiling, during PCa progression in the genetically developed PTEN mouse model.

Subtask 1: Development of PCa mouse model and determination of altered expression of miRNAs in PCa progression.

In order to investigate the altered global expression profiles of miRNAs during PCa progression in mice having PCa with/without castration. Briefly, Dr. Batra's group (Project mentor) has already generated *Pten$^{fl/fl}$;Pb-Cre/;p53R172H;Pb-Cre4^{+}* mice that spontaneously develops PCa. In our observation, we found that mice of 14-16 weeks of age started developing tumor in prostate. Hence, after genotyping, we divided mouse into two groups i.e. first group consisting of mice shown positivity for the lox p sites in the PTEN gene, mutatedR172H, Rosa26 and probasin-Cre and 2nd groups with only wild type PTEN.. We euthanized three mice in each group of 16 weeks of age (postnatal).. Malignant and normal prostate tissues were excised and made into two parts. A part of the prostate was flash-freshened in liquid nitrogen for RNA isolation and the other part is fixed in buffered formalin for histological and pathological analysis.



Scheme 2: validation of altered level of miRNAs in PCa tissue and patient serum compare to respective controls. Figure-2 (A) TaqMan assay for validation in altered miRNA levels in PCa mice tissue. Exp. outcomes revealed miR-21, miR-375 and miR-141 are differentially upregulated in PCa tissue excised from the Pten conditional knockout mice harboring p53 mutation, compared to their littermate and internal age match controls.Figure-2 (B) TaqMan assay showing relative fold change in miR-21 expression level in the sera of PCa patient (n = 4) compare to normal controls. Significant overexpression of miR-21 level observed in cancer patient compare to controls.

Furthermore, miRNAs/total RNAs (T-RNAs) were isolated from flash frozen PCa and normal tissue sample using MirVana RNA isolation kit (Thermo Scientific). We performed TaqMan assays (Applied Biosystems) using probes specific for miR-21, miR-141 miR-375, miR-210, miR-221, miR-155 and let-7b in those isolated miRNAs. These miRNAs were selected based upon their significant association in human PCa. Further, outcomes from Taq-Man analsysis on PCa mouse tissues and PCa patient serum shown significant overexpression of miR-21 along with increased expression of miR-375 and miR-141 too in PCa mouse model. Hence, finally we chose miR-21 as a model for sensitive detection using a DNA-Au nanoprobe-based kinetic assay.

Subtask 1: DSN mediated miRNA quantification assay via DNA-Au nanoprobe

Nanoprobe assay: To begin with our actual serum based assay, initially we have performed standardization of our nanoprobe-based fluorescent assay protocol used in miRNA quantitation. These preliminary analysis was demonstrated using different concentrations (Range 50 pM – 10 nM) of synthetic miR-21 (IDT-DNA Technologies) as shown in following figures (3A and 3B). Finally, we have also studied specificity and sensitivity of our nanoprobes in miRNA detection.

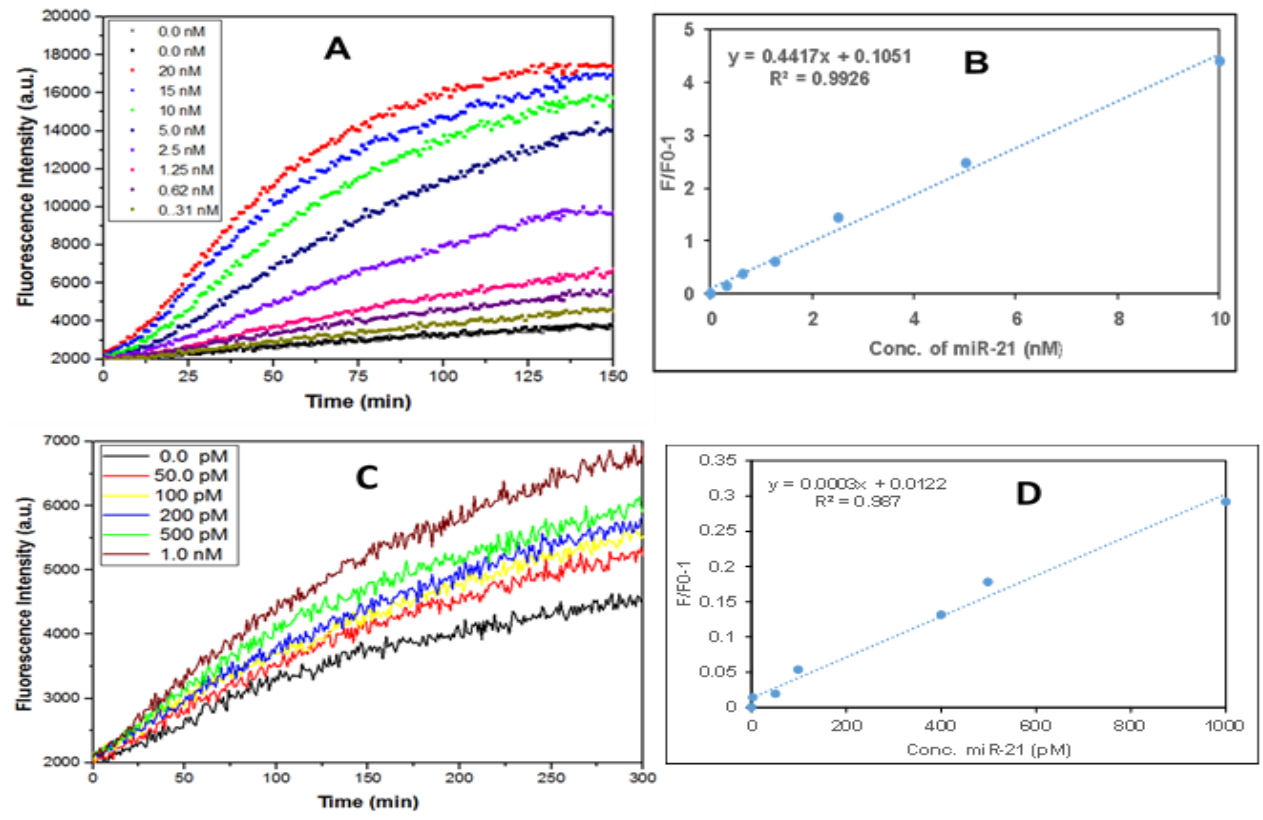


Figure-3: Real-time monitoring of assay mixtures containing different concentrations of synthetic miR-21). Figure (A and C) kinetic release of fluorescence signals with DSN treatment, synthetic miRNA conc. ranges between (0.3 nM nM) showing a very good linear response curve as observed in figure B and D respectively

Study of sensitivity and specificity of Au nanoprobe: As a conclusion, we achieved we achieved a sensitive detection of synthetic miR-21 via DNA-Au nanoprobe with the lower limit of detection (LoD) of 50 pM, displaying a linear relation between [miR-21] with R2 value = ~ 0.99. Analysis of detection specificity of assay with original (Fig. 4A) and artificially introduced mutations (Fig. 4B) including in the seed region of synthetic miR-21 sequence. Comparing both Fig. 4A and 4B, displaying high specificity of detection probe for miR-21. Our assay is clearly capable of discriminating specific target from mismatched strand. The sequence specificity have also seen despite the use of unnatural/artificial miRNAs.

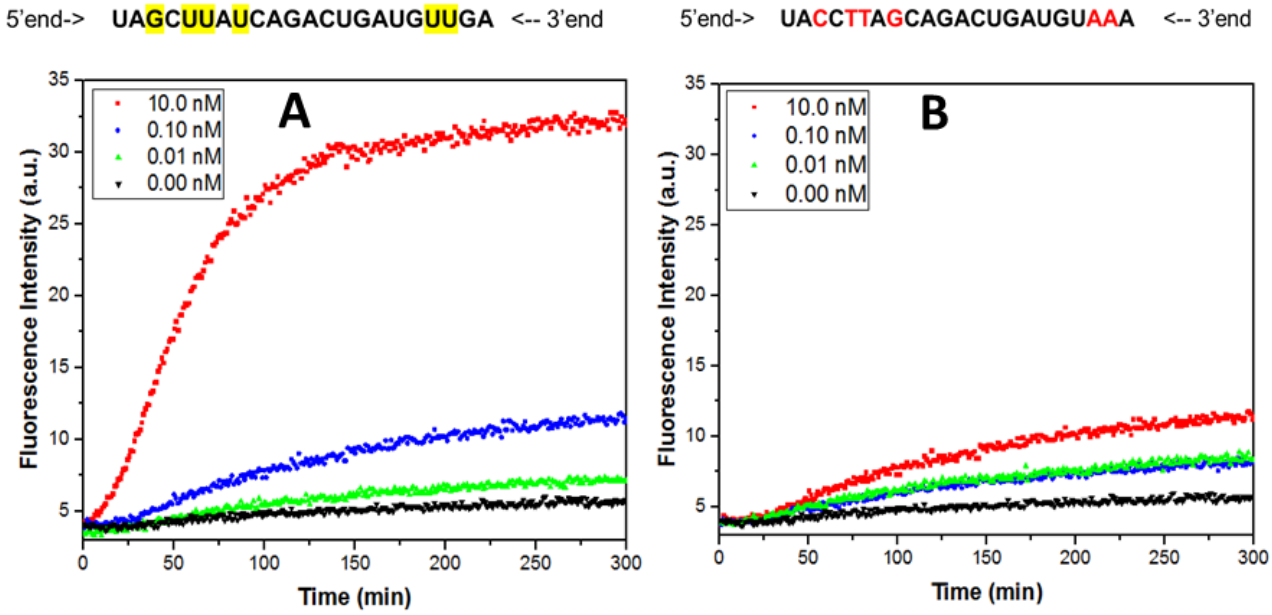


Figure-4: Analysis of detection specificity of assay with original (Fig. 4A) and artificially introduced mutations (Fig. 4B) including in the seed region of synthetic miR-21 sequence.

DNA-Au nanoprobe-based quantification of miR-21 in mouse PCa tissue/Human serum

As proof of principle, we initially confirmed our assay strategy is working with isolated total RNAs from mouse PCa tissues and serum samples (Figure). DNA-AuNP-based kinetic assay revealed that miR-21 expression fold change was higher in PCa patients compared to controls. Currently several biomarker detecting nanoprobe have been discovered for miRNAs or other nucleic acids. However, a real application of these nanoscale probes on real/patient serum sample is still limited or not much explored. Hence, we collectively displayed a quantitative analysis of normal vs PCa serum sample taking maximum number of serum sample. Here, we have successfully developed a liquid phased, DNA-AuNP-based, kinetic assay for direct, single step, sensitive (LoD = 50 pM, 0.64 fmol),

sequence specific quantification of synthetic miR-21 as well as miRNAs isolated from biological samples. In future, our goal to explore these assay to differential indolent vs. aggressive disease and achieving multiplexed detection of miRNAs at a time.

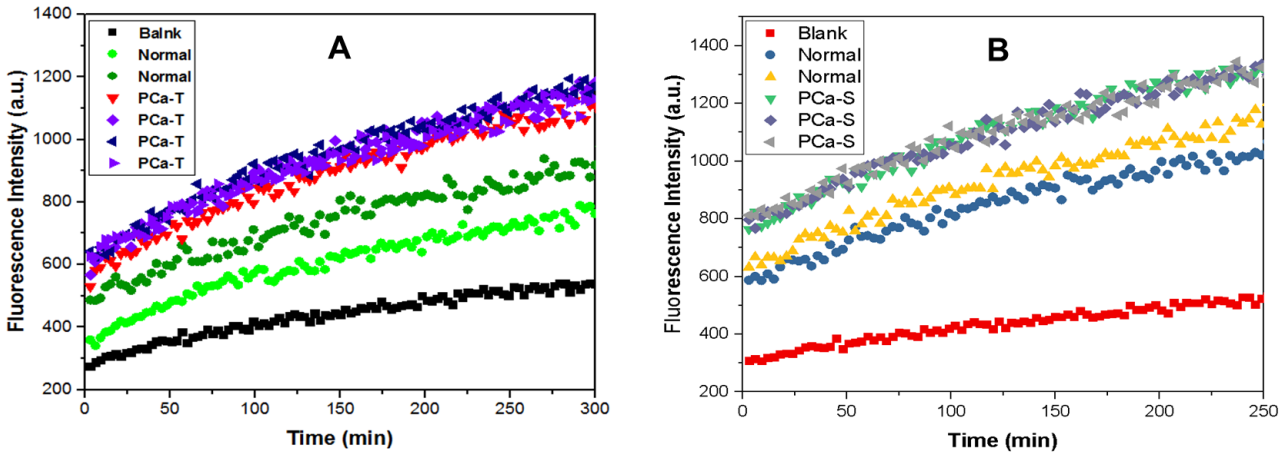


Figure-5: Detection of miR-21 in total RNAs isolated from (A) PCa mouse tissues and (B) sera of PCa patient samples including respective age-matched controls.

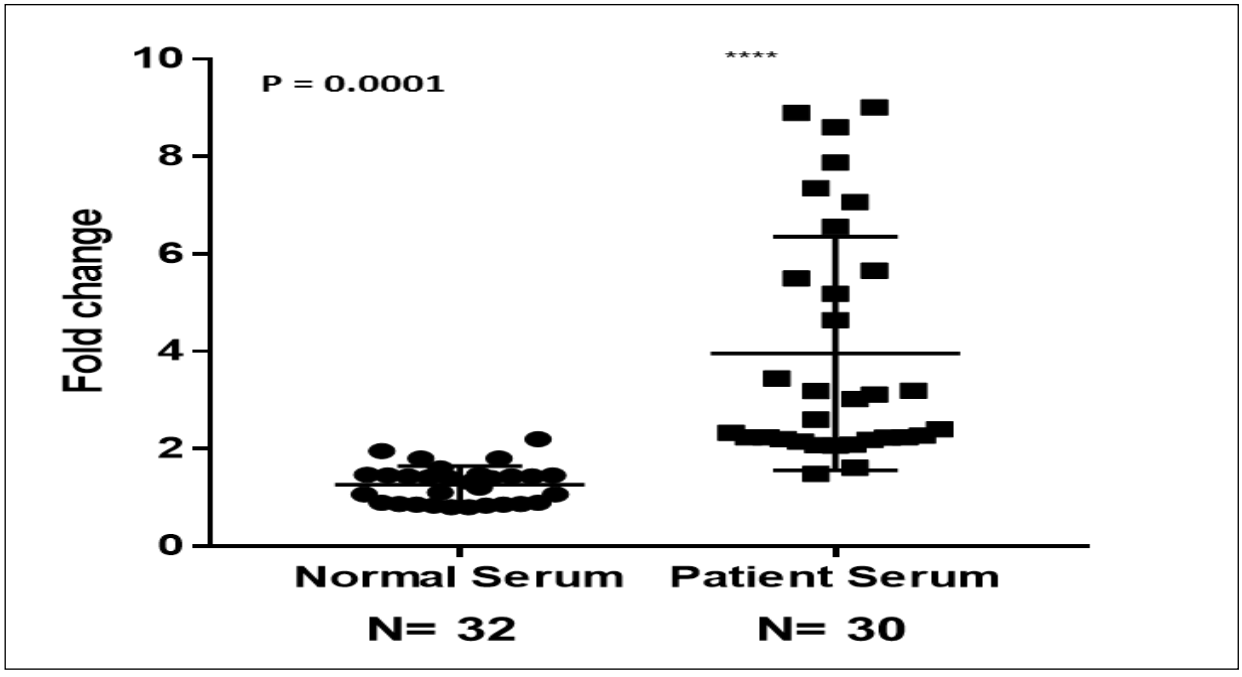


Figure 6: Detection of miR-21 in total RNA from normal and PCa serum sample, showing upregulation of serum miRNA-21 in PCa patient compare to normal one.

Where the results disseminated to community of interest?

"Nothing to report"

Plan for next reporting period to accomplish the goal

Plan-1: Successful development of multiplexed DNA-nanoprobe and set-up quantification protocol for mouse model: Based on the current outcomes, we will standardized same procedure for patient serum.

Plan-2: Ultimate aim for differentiation of indolent vs aggressive PCa through via multiplexed nanoprobe assay, which will be verified with patient medical history, PSA level and other cancer signature.

4) Impact:

Impact on the development of the principle discipline of the project

The proposed grant studies in PCRP-FY2104 postdoctoral fellowship application will help to launch innovative nanotechnology based sensitive nanoprobes as a diagnostic tools for early detection of aberrant expression of biomarker miRNAs. Furthermore, our studies will investigate stage specific global miRNA expression profiles in the PCa tissue during murine PCa progression (Pten; p53; Pb-Cre PTEN model readily available in mentor's lab) followed by their detection in mouse serum and in sera of human prostate cancer patients by using innovated nanoprobes. On the basis of current outcomes, we are confident that, the current proposal and our future studies will give results into the development of effective strategies for early detection of novel oncogenic miRNAs using sensitive Au based nanoprobes. Subsequently results into an effective diagnostic tools for PCa diagnosis as well as differentiate between androgen dependent to androgen independent PCa in patient. Overall the project will lead to the discovery of novel circulating biomarkers and development of novel detection technologies which in combination will be useful in distinguishing indolent vs aggressive and hormone dependent vs independent PCa. Hence our proposal addresses and aligns with the PCRP-FY2014 focus area "Development of Biomarkers for PCa and their early detection for accurate and on-time diagnosis".

Impact on other disciplines and technology transfer

Nothing to report. (Currently we have finished with developing the Multiplexed nanoprobe for quantification miRNA in PCa..

Impact on society beyond science and technology

Prostate cancer (PCa) is the most commonly diagnosed and second leading cause of cancer related mortality in US men. There is a lack of sensitive and specific technology resulting into the high incidence of patient death worldwide. All these advanced

nanotechnology-based research strategies will make a huge impact on early stage diagnosed PCa patients by providing them early stage medication/therapies and by contributing to the goal of eliminating death risk. It will be one step close towards the eradication of PCa from the society.

5) Changes/Problems

Nothing to report

6) Product

1) Review article: Currently we are in the process of writing a review article entitled- "Advanced Nanotechnology based Detection of Cancer Biomarkers"

2) Presentation: Presented in UNMC-SPORE retreat held at Nebraska city during (1st June – 3rd June 2016), seminar entitled "Direct and Absolute Quantification of micro-RNA (miRNA) using AuNP-*DNA (Off-On) Probe"

3) Attending incoming the 14th International Nanomedicine & Drug Delivery Symposium (nanoDDS'16) will be held at The Johns Hopkins University, Baltimore, Maryland, from September 16-18, 2016.

4) Advanced DNA-gold nanoprobe-based direct and sensitive quantification of novel microRNAs in prostate cancer
Cancer Research 77(13 Supplement) LB-331-LB-331 · July 2017
DOI: 10.1158/1538-7445.AM2017-LB-331

5) DNA-Au nanoprobe based detection of miRNAs in detection of prostate cancer. (Article under preparation)

7) Participant and Other Collaborating Organization

Nothing to report

8) Appendices

Nothing to report

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